LESSON 27: PLANT TISSUE CULTURE AND GENETIC TRANSFORMATION

Introduction

One of the most recent areas of plant cell and tissue culture developed for exploring potential benefits in agriculture has been the transfer and expression of foreign genes into plant cells. The transformation of bacterial cells by uptake of DNA from another bacterium, and subsequent integration of this foreign DNA into the genetic material of the host is well established. However, genetic modification of higher plants by introducing DNA into their cells is a highly complex process. For a cell to be transformed permanently, a foreign nucleotide sequence has to be introduced in such a way that alien genetic information should be able to express in the transformed cells.

Availability of restriction endonucleases (which cut duplex DNA molecules into discrete fragments), development of the DNA-DNA hybridisation technique and marker genes for selection of transformed cells have now enabled incorporation of exogenous DNA into cells of Monera, fungi, animals, and higher plants. Recent studies have shown that new genetic information added as a result of these processes in eukaryotic plants expresses not only at the cellular and later, whole organism level, but also can be transmitted in successive generations of transformed individuals. Such transgenic plants are not known to exist in nature since normally they originate following an exchange of genetic information between sexually incompatible species.

In this chapter the systems and methodology used for genetic transformation of higher plants are briefly summarised. An attempt is made to assess the fate of foreign genes in transgenic plants and their application to agricultural biotechnology.

Transformation through Uptake of Foreign DNA by Seeds and Seedlings

The pioneering work on genetic modification of plants by introduqing isolated DNA was done with whole plant systems such as seeds and seedlings. These studies seemed to reveal that exogenously applied DNA is not only taken up by plants, but also integrate into the host genome. The evidence for integration and replication of the exogenous DNA was based mainly on isopycnic centrifugation in cesium chloride (CsCl) gradients.

Two major types of experiments, referred to as integration and replication, were conducted. In the integration experiment, radioactive labelled DNA of density different from the host DNA was administered to the seedlings. This was followed by isolating DNA from seedlings, or their excised parts, after a period of metabolism for analysis on CsCl gradients. Evidence for integration of the radioactive donor DNA was ascertained by the occurrence of a radioactive band approximately intermediate in density between the host and donor DNA. The intermediate density band was further analysed by sonication and denaturation. Experiments of this type demonstrated the 'integration' of Micrococcus fysodeikticus DNA into barley root, E. coli and Streptomyces coelicolor DNA into Arabidopsis and T4 DNA into Matthiola incana seedlings. The replication experiments were also conducted in a similar manner and differed only in respect of the donor DNA, which was non-radioactive. These experiments also showed evidence of bacterial plant DNA complexes in tomato, barley and Arabidopsis.

Several workers have tried transformation experiments using a similar donor DNA with the same host or different plant systems but were unable to reproduce the same results. They concluded that there was neither integration nor replication of the exogenous DNA inside the host cells. In fact, the donor DNA ultimately degraded in the host and the degraded products were used for endogenous DNA synthesis. Regarding the claim that exogenous DNA could also be taken up by seeds was probably accounted for by adsorption of DNA on the cell walls and cell membrane.

A new thrust, however, on genetic transformation of whole plant systems seems to have developed recently as a result of the success achieved with cereals. The reported rhizobial infection of non-legumes to form an effective nodular nitrogen-fixing symbiosis within the dicot plants belonging to the genus Parasponia (Trinick 1982) has generated such interest as to extend nodular symbiotic association to monocots, especially the cereals. Cocking and co-workers (Al-Mallah et al. 1989) reported induction of nodular structures on the roots of rice seedlings when these roots were treated with a cellulase-pectolyase enzyme mixture followed by inoculation with either Rhizobium, or Bradyrhizobium, in the presence of PEG. This first report of inducing nodular structure on a rice plant by Rhizobia in vitro has opened new vistas in the genetic engineering of plants as it may be possible to extend this technique for effective nodulation in respect of other cereals as well as nonlegumes. Further investigations are required to determine whether nodular structures on transformed rice are effective in nitrogen fixation. Otherwise, other strains of genetically engineered Rhizobium, or its wild type, may be needed for this purpose.

Uptake of DNA by Pollen

In 1976, Hess and co-workers incubated Nicotiana glauca pollen with DNA isolated from Nicotiana langsdorffii and then used this pollen to pollinate emasculated N. glauca flowers. They reported that this experiment led to the production of transformed plants which developed tumours at the wound site on the stem. This is a characteristic feature exhibited by the sexual hybrids arising between these two species. Ohta (1986) mixed pollen from maize plant homozygous for aleurone and various endosperm marker colours with DNA extracted from a strain homozygous dominant for all these traits. They achieved a high pollen transformation efficiency. These observations suggest that pollen may act as vehicles for the transfer of foreign genes into plants.

In another approach, de la Pena et al. (1987) manually injected young floral tillers of rye plant with a solution of plasmid DNA carrying a chimeric gene consisting of T-DNA nopaline synthase gene flanking coding sequence of the Tn 5 neo gene (expressing for antibiotic aminoglycoside resistance). Injected flowers were pollinated with normal pollen and progeny seeds plated on kanamycin medium. Of the 3000 seeds, only two seedlings were isolated showing traits of kanamycin resistance as well as the nos-neo gene. At the time of injection the male germ cells lacked a surrounding callose wall and hence the authors concluded that the injected DNA was taken up by male gametes. However, this method needs wider applicability as it would bypass all the problems associated with plant cell or protoplast cultures by enabling the direct recovery of transgenic plants from gametes treated in situ.

Transformation of Protoplasts

The isolated protoplast system has proved most promising for genetic modification of cells. In the absence of a wall around the plasma membrane, protoplasts are not only able to fuse but can also take up chloroplasts, nuclei, micro-organisms and isolated foreign DNA. This feature has prompted workers to investigate various modes of genetic modifications in cells using the protoplast system.

Uptake of Organelles

Chloroplast Transplantation

Several workers reported the uptake of chloroplasts by albino protoplasts isolated from Petunia, tobacco and carrot plants during the years 1973-1974. This required a simple treatment-exposing a mixture of albino protoplasts and chloroplasts to PEG in a manner similar to protoplast fusion. EM studies revealed that PEG caused fusion of the two external membranes of the plastids and while entering the protoplasts both the plastid and protoplast membranes fused, causing disruption of these organelles (Davey et al. 1976). In view of this, there was a shift in emphasis from direct uptake to transfer of chloroplasts through the fusion of protoplasts. Using this approach it could be possible to restore photo-autotrophy in albino protoplasts by fusion with wild type (green) protoplasts in a number of species (see Giles 1989). Medgyesy et al. (1985) used a gamma-irradiated chloroplast-mutant of Nicotiana species for interspecific chloroplast transfer by protoplast fusion and in the process produced cybrids, whereas Cseplo et al. (1986) demonstrated that protoplast fusion was suitable to rescue the chloroplast mutants of N. plumbaginifolia from undesirable nuclear backgrounds.

The practical value of chloroplast transformation via protoplast fusion, in plant improvement could be demonstrated by transfer of genes for herbicide resistance between related species. For example, isolation of mutants by cell selection in populations of protoplast-derived colonies yielded triazine-resistant Nicotiana plants. The resistant trait was observed to arise as a result of the mutation in the chloroplast gene psb-A. Similarly, inhibitors of bacterial protein synthesis were used to induce chloroplast mutations in protoplast cultures of N. plumbaginifolia. In the process it could be possible to isolate mutants resistant to antibiotics and photosynthesis-inhibiting herbicides (Cseplo et al. 1986). The bacterial APH (3) II gene from transposon Tn 5 (which confers resistance to the related amino glycoside antibiotics, neomycin, kanamycin, and G418) and the cat gene from transposon Tn 9 (which confers resistance to chloramphenicol, an inhibitor of protein synthesis on 70S ribosomes) are selectable marker or reporter genes that have been found suitable for transfer of mutant chloroplast genes (Gray 1989).

Fluhr (1989) recently described how the interaction between chloroplast genes from weeds to sexually incompatible crop plants results in the control of light-mediated proteins expressed by rbcs and cab gene families. The transfer of chloroplast genes from weeds to sexually incompatible crop plants is another area that may have potential in future. Thus, studies on chloroplast uptake and exchange have played a useful role in understanding the nuclear-chloroplast interaction, physiology of isolated plastids and transfer of herbicide resistance.

Mitochondrial Transplantation

There is no evidence regarding the direct uptake of isolated mitochondria by protoplasts. However, there are reports on incorporation of mitochondrial DNA (mt DNA) through protoplast fusion leading to the formation of cybrids. Motochondrial genomes were first analysed in Nicotiana cybrids (Belliard et al. 1979) and, subsequently, in other cybrids produced by somatic hybridisation. It may also be possible to transfer traits such as toxin sensitivity of Texas male sterile cytoplasm encoded by mt DNA in maize (Lonsdale 1987).

Nuclear Transplantation

Potrykus and Hoffmann (1973) reported uptake of isolated nuclei by mesophyll protoplasts of Petunia, tobacco and maize. Transplantation success improved from 0.5% to 5% in the presence of PEG and Ca++. Since then the methods for isolation and uptake of nuclei by protoplasts have been refined to facilitate expression of the transferred genome in the cells. Saxena et al. (1986, 1987) transplanted Vicia hajastana nuclei in protoplasts of an auxotrophic cell line of Datura innoxia and isolated prototrophic cell colonies expressing for Vicia genomic DNA in cultures. However, these colonies on an appropriate regeneration medium developed shoots typical of wild type Datura.

The uptake of isolated chromosomes, instead of complete nuclei, may be another area to explore for transfer of foreign genes into plant cells. Development of the flow cytometry technique enabled the introduction of isolated chromosomes into protoplasts of Haplopappus (De Laat and Blaas 1984), Nicotiana (Verhoevenet al. 1987) and Petunia (Conia et al. 1987). Only metaphase chromosomes were found suitable. Isolated chromosomes can also be introduced into protoplasts by microinjunction (De Laat and Blaas 1987). In most of these experiments transplanted chromosomes were eliminated due to incompatibility.

Transfer of micronuclei into protoplasts has also been attempted. Amiprophos-methyl (APM) treatment of potato and carrot cells promoted accumulation of a large number of metaphases. The scattered single or groups of chromosomes in these cells developed a nuclear membrane and formed micronu-

clei which could be sorted out by flow cytometry and then used for transplantation (Sree Ramulu et al. 1987). This opens prospects for application of micronuclei for transferring specific intact chromosomes for the purpose of gene mapping.

Uptake of Micro-organisms

Initial attempts to transfer whole bacteria (Rhizobium) into protoplasts with a view to utilising the transformed protoplasts for nitrogen fixation yielded little success. Therefore, the emphasis shifted to the use of viruses as vectors for genetic engineering studies. Studies on aspects such as mechanism of infection, nuclear-protein interaction, integration and expression of viral DNA in isolated protoplasts should be possible since in nature viruses do infect plants and multiply within their tissues. In early studies, transducing phages were used to transfer bacterial genes into plant cell lines or callus tissues. Although bacterial genes appeared to express briefly in the plant cells or callus cultures treated with phages, there was no evidence of "transgenosis" (asexual transfer, expression, and inheritance of genetic information between donor and recipient organisms separated by evolution) in long term cultures (Kleinhofs and Behki 1977).

Experiments on uptake of TMV by isolated protoplasts were initiated in 1969 (Cocking and Pojnar 1969, Takebe and Otsuki 1969). Since then, numerous reports have appeared on the incorporation of viral nucleic acids into protoplasts and using them as vectors in genetic transformation. Most plant viruses have encapsulated genomes and it is assumed that no specific virus receptor sites exist on the exterior of the plasma membrane. Viral based transforming agents may be of the integrating or non-integrating type.

In selecting the progenitor (virus) for the transforming agent, the factors generally considered are: (a) amenability to genetic engineering techniques, (b) packaging constraints in the particle and (c) development of a system in which expression can be studied. The cauliflower mosaic virus (CaMV) is the most amenable and widely chosen progenitor for genetic engineering studies. Moreover, the dsDNA genome in CaMV has appropriate restriction sites compatible with the methodology developed for Agrobacterium tumefaciens plasmid (Ti plasmid). Brisson et al. (1984) were successful in introducing a chimeral CaMV carrying methotrexate resistance into turnip cells in such a way that the virus replicated normally in the transformed plants. Similarly, barley protoplasts infected with the ssRNA bromovirus (BMV), on having the chloramphenicol acetyltransferase (CAT) bacterial gene inserted into the RNA3 genome, transcribed for CAT activity (French et al. 1986). Geminiviruses may also be used for genetic transformations but their usefulness as vectors are still under study.

Another novel approach under consideration in genetic engineering is the Agroinfection which involves a combination of viral and bacterial vectors. Various strategies developed for uptake of viruses by plant protoplasts and their use as transforming agents have been reviewed by Cassells (1989).

Use of fungal protoplasts as eukaryotic gene vectors for plant cell transformation has also been attempted. The uptake of fungal protoplasts requires a suitable experimental system since fungi and higher plants establish a range of nutritional relationships (heterotrophic, saprotrophic, symbiotic etc.) in nature. Constabel et al. (1982) fused Catharanthus roseus protoplasts with fungal protoplasts in an attempt to transfer the secondary metabolite synthesising ability of fungus into a higher plant protoplast system in cultures. Later, Lynch et al. (1989) successfully mediated uptake of celery (Apium graveo/ens) protoplasts by Aspergillus nidu/ans and Fusarium oxysporium protoplasts using a PEG solution, which was confirmed by ultrastructural studies and diamidino-2-phenylindole staining. Protoplasts of celery showed improvement in the level of viability after uptake by A. nidu/ans, whereas with F. oxysporium the level decreased. However, it remains to be seen whether uptake of fungal protoplasts can be exploited for secondary metabolite synthesis in higher plant cell or protoplast system.

Transformation Using Agrobacterium System

The effort expended in plant genetic transformation appeared to be in vain since studies using various systems for transfer of exogenous DNA into the genome of higher plants failed to give reproducible results. In the past few years, however, molecular studies of crown gall disease by Agrobacterium plasmids and the use of recombinant DNA technology, have renewed interest in the subject and further investigations have been carried out using Agrobacterium for plant genetic manipulations.

What is an Agrobacterium?

Agrobacterium tumefaciens, a soil bacterium, has the ability to infect most dicotyledonous plants, usually at a wound site. The tissue around the infected wound develops a neoplastic growth known as a crown gall tumour. The tumour tissue excised from the plant is capable of growing on hormone free medium independent of the bacterium. In cultures, the crown gall tissue produces a set of metabolites termed opines (amino acids or sugar derivatives which plant cells do not metabolise normally but which can be utillsad as a carbon and nitrogen source by the A. tumefaciens strain responsible for induction of the tumour). Agrobacterium harbours the Ti plasmid, which is directly responsible for tumour induction. The transfer of small DNA segments from this plasmid and their integration into the genome of host cells induces tumour formation in the plants. The property of the Ti plasmid DNA segment (T-DNA) integrating with the genome of higher plants has attracted world-wide attention for using Agrobacterium in genetic transformation techniques.

Agrobacterium rhizogenes is another bacterium used in plant genetic manipulations. The infection by this bacterium causes hairy roots which are of clonal origin. Unlike A. tumefaciens tumours, the tumor produced by A. rhizogenes has the capability for regenerating mature fertile plants. The tumour-inducing properties of rhizogenes strains are also carried on a large Ri plasmid DNA (Ri T-DNA). Two segments of this DNA (TL-DNA and TR- DNA) are transferred to plant cells during infection by A. rhizogenes.

Conclusion

Practically any plant transformation experiment relies at some point on cell and tissue culture. There are some exceptions to this generalisation, but the ability to regenerate plants from isolated cells or tissues in vitro underpins most plant transformation systems. Some plant species may be amenable to regeneration by a variety of integration of plant tissue culture into plant transformation protocols. Not all plant tissue is suited to every plant transformation method, and not all plant species can be regenerated by every method. There is therefore a need to find both a suitable plant tissue culture/regeneration regime and a compatible plant transformation methodology.

Questions

1. Discuss the strategies followed for transformation of plant cells.
2. What is an agrobacterium?

Note